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Article

An Essential Role for the NLRP3 Inflammasome in Host Defense against the Human Fungal Pathogen *Candida albicans*

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SUMMARY

Candida albicans **is an opportunistic fungal pathogen causing life-threatening mucosal and systemic infections in immunocompromised humans. Using a murine model of mucosal *Candida* infection, we investigated the role of the proinflammatory cytokine IL-1β in host defense to *Candida albicans.* We find that the synthesis, processing, and release of IL-1β in response to *Candida* are tightly controlled and first require transcriptional induction, followed by a second signal leading to caspase-1-mediated cleavage of the pro-IL-1β cytokine. The known fungal pattern recognition receptors TLR2 and Dectin-1 regulate IL-1β gene transcription, whereas the NLRP3-containing proinflammatory multiprotein complex, the NLRP3 inflammasome, controls caspase-1-mediated cleavage of pro-IL-1β. Furthermore, we show that TLR2, Dectin-1, and NLRP3 are essential for defense against dissemination of mucosal infection and mortality in vivo. Therefore, in addition to sensing bacterial and viral pathogens, the NLRP3 inflammasome senses fungal pathogens and is critical in host defense against *Candida*.**

INTRODUCTION

Opportunistic fungi cause life-threatening infections in immunocompromised hosts. In healthy individuals, *Candida spp.* have low pathogenicity; however, with depressed host immunity or when changes occur in oral microbial flora, overgrowth can occur, resulting in oropharyngeal candidiasis (OPC, also known as thrush) or denture stomatitis (Abu-Elteen and Abu-Alteen, 1998; Daniluk et al., 2006; Figueiral et al., 2007). *Candida albicans* is the leading cause of life-threatening fungal disease and ranks fourth among all bloodstream and nosocomial infections in the United States (Jarvis et al., 1995; Wisplinghoff et al., 2004).

Considerable progress has been made in recent years in our understanding of how pathogens are recognized by the innate immune system and how sensing translates into signaling and transcriptional regulation of immune response genes. Several classes of germline-encoded pattern recognition receptors (PRRs) have now been implicated in innate defenses. These include the Toll-like receptors (TLRs) (Takeda and Akira, 2005), the C-type lectin receptors (CLRs) (Huysamen and Brown, 2009), the RIG-like helicases (RLRs) (Yoneyama and Fujita, 2007), cytosolic DNA sensors (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009; Takaoka et al., 2007), and members of the NOD-like receptor (NLR) family (Meylan et al., 2006). Recent evidence has implicated TLR2 and the CLR family member Dectin-1 in fungal recognition, phagocytosis, and the induction of inflammatory responses (Gantner et al., 2003; Herre et al., 2004; Kerrigan and Brown, 2009; Rogers et al., 2005).

IL-1β is a proinflammatory cytokine released by many cell types and is an important mediator of inflammation during infection and inflammation (Dinarello, 2002, 2005). The synthesis, processing, and release of IL-1β are tightly controlled and require at least two distinct stimuli. An initial microbial stimulus through innate PRRs results in accumulation of intracellular stores of pro-IL-1β. A second stimulus activates a multiprotein complex containing one or more NLRs, commonly referred to as the "inflammasome," which controls the activation of caspase-1 and cleavage of pro-IL-1β, followed by release of the active mature 17 kDa IL-1β (Franchi et al., 2009; Meylan et al., 2006). The NLRs consist of a C-terminal LRR domain, a central nucleotide-binding oligomerization (NACHT) domain, and a variable N-terminal protein-protein interaction domain that can be either a CARD, Pyrin, or BIR domain (Franchi et al., 2009; Martinon and Tschopp, 2005). The best characterized inflammasome complex contains NLRP3, ASC (apoptosis-associated speck-like protein), CARDINAL, and caspase-1 (Amer et al., 2006; Franchi et al., 2006; Miao et al., 2006, 2008; Sutterwala and Flavell, 2009; Sutterwala et al., 2007). The NLRP3 inflammasome is activated by many microbial stimuli, including bacterial pore-forming toxins (Mariathasan et al., 2006; Martinon et al., 2004), by viruses such as Sendai virus (Kanneganti et al., 2006a), influenza (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009), and adenovirus (Muruve et al., 2008) and by endogenous danger signals such as ATP and monosodium urate (Martinon et al., 2006; Martinon and Tschopp, 2004). Additional activators include indigestible particulates like silica, asbestos, alum, and β-amyloid (Dostert et al., 2008; Eisenbarth et al., 2008; Halle et al., 2008; Hornung et al., 2008). Although roles for IL-1α/β

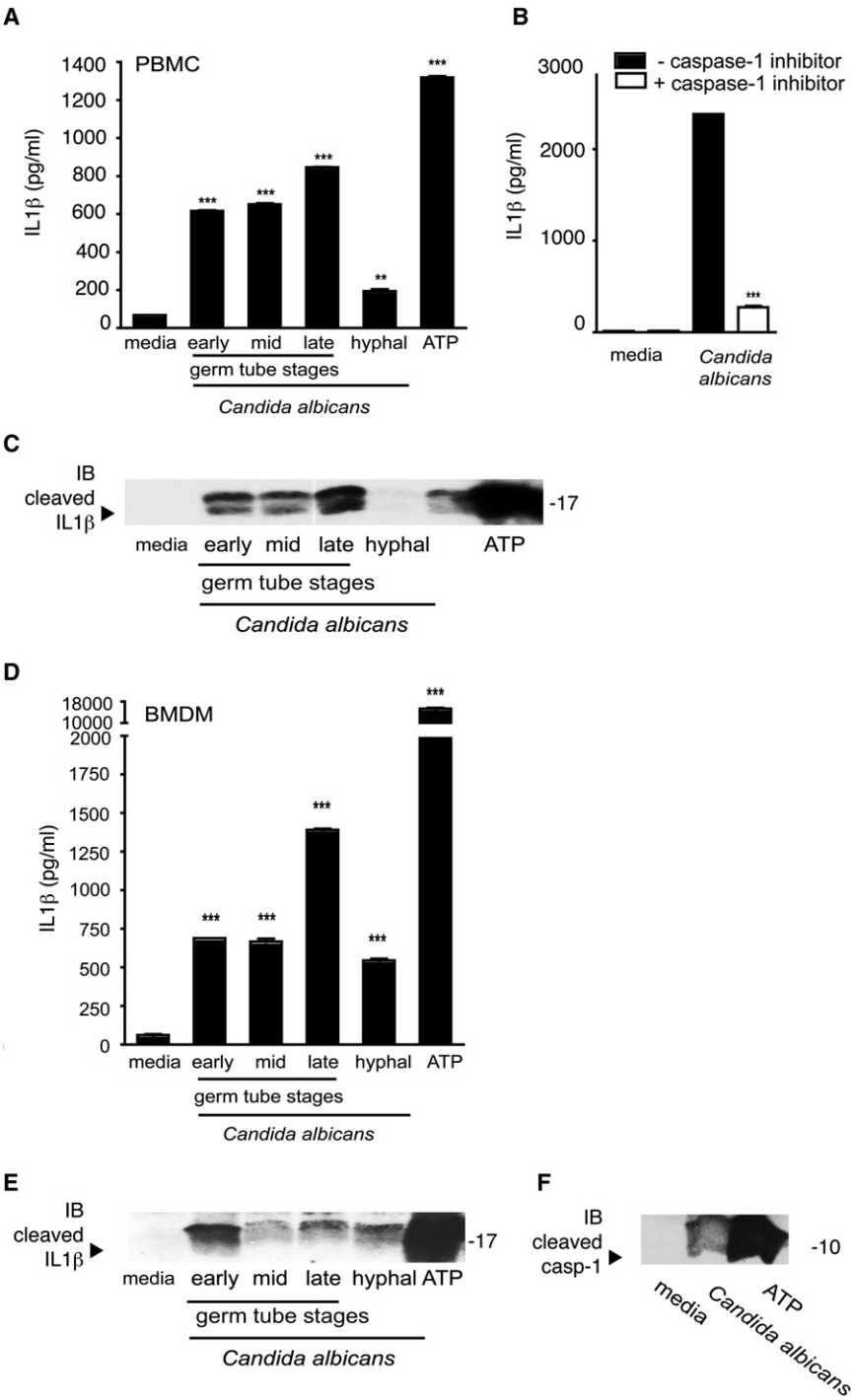

Figure 1. In Vitro IL-1β Responses to *Candida albicans* Are Morphological Stage Dependent and Blocked by Caspase-1 Inhibitor

(A) IL-1β responses of normal PBMC primed for 3 hr with LPS and then stimulated with fixed preparations of *C. albicans* of different morphological stages at ~10^6/ml.

(B) Inhibition of IL-1β responses after the addition of caspase-1-specific inhibitor, Z-YVAD-FMK.

(C) Western blot of supernatants from PBMC stimulation probed with anti-IL-1β antibody.

(D) IL-1β responses of bone marrow-derived macrophages from wild-type mice primed for 3 hr with LPS and then stimulated with *C. albicans* at ~10^6/ml.

(E and F) Western blot of supernatants from BMDM stimulation probed with (E) anti-IL-1β antibody and (F) anti-caspase-1 antibody.

***p < 0.001; **p < 0.01; *p < 0.05. Error bars, ± SD.

and IL-18 have been shown in some animal models of fungal infection (Bellocchio et al., 2004; Mencacci et al., 2000; Netea et al., 2002; Steele and Fidel, 2002; Stuyt et al., 2002, 2004; Vonk et al., 2006), the role of NLRs and inflammasomes in host defense to fungal pathogens has not been addressed.

Here, we investigate the role of IL-1β in host defense to *Candida albicans* by using a murine model of sustained mucosal infection. We show that TLR2 and dectin-1 control IL-1β gene transcription, whereas NLRP3, ASC, and caspase-1 regulate the processing of pro-IL-1β into the active mature 17 kDa cytokine. Importantly, we also reveal a critical role for TLR2, as well as Dectin-1 and the NRLP3 inflammasome, against disseminated infection and mortality in vivo. Therefore, our results implicate the NLRP3 inflammasome and IL-1β production in the regulation of mucosal antifungal host defenses.

RESULTS

Candida albicans Activates a Caspase-1-Dependent IL-1β Response, which Is Dependent on the Morphological Stage of the Organism

Candida spp. are dimorphic fungi that grow as yeast or hyphal forms, depending on environmental conditions. We first examined IL-1β production by different morphological forms of *C. albicans*. Human PBMC were exposed to formalin-fixed preparations of *C. albicans* grown to germ tube stage (exposed to hyphal-permissive media for 2, 4, or 6 hr, respectively) or fully hyphal forms (>24 hr growth). As shown in Figure 1A, *Candida* induction of IL-1β from LPS-primed PBMC was strongest in response to germ tube forms and comparable to ATP-treated controls. Indeed, in cells that were incubated with *Candida* in the presence of the specific caspase-1 inhibitor Z-YVAD-fmk, a synthetic peptide that irreversibly and specifically inhibits caspase-1 activity, almost complete inhibition of IL-1β release was observed (Figure 1B). Similar data were obtained when THP-1 cells were examined (Figure S1 available online). We also tested the presence of IL-1β in cellular

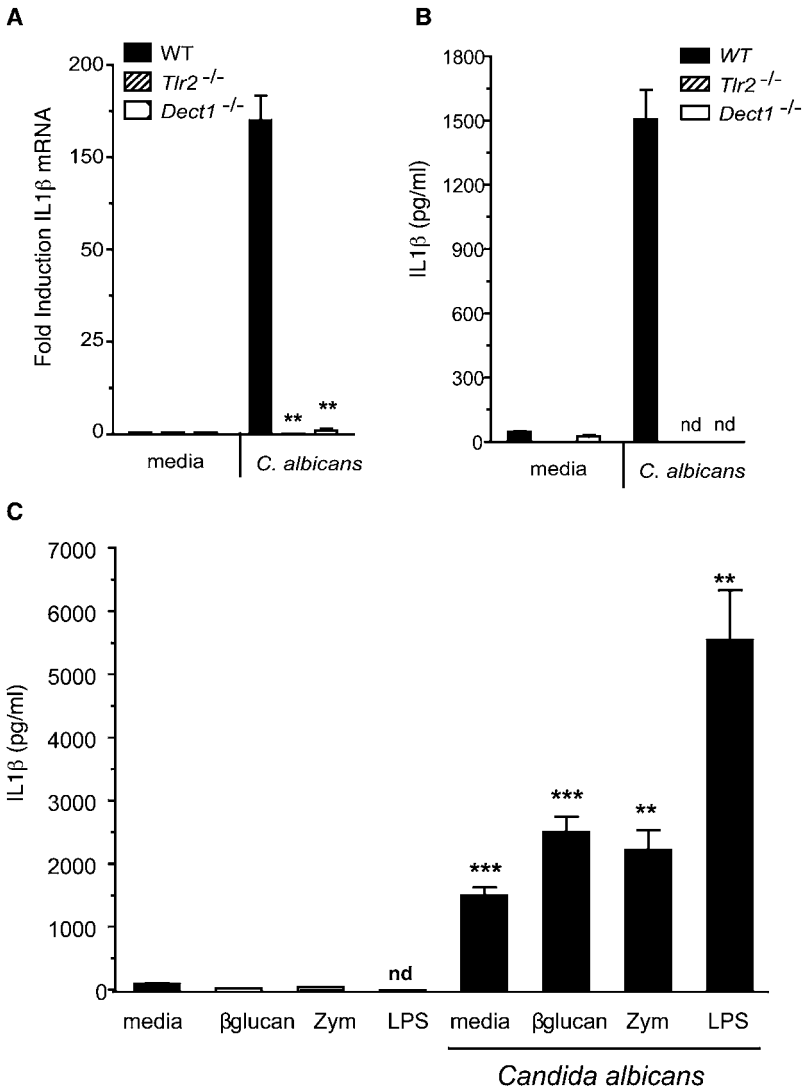

Figure 2. IL-1β Responses to *Candida albicans* Are Dependent on TLR2 and Dectin-1

(A) Upregulation of IL-1β mRNA by qPCR of macrophages from *Tlr2*$^{-/-}$, *Dect1*$^{-/-}$, and wild-type mice stimulated for 8 hr with *C. albicans* of different morphological stages at ~10^6/ml.

(B) IL-1β protein levels in supernatants of macrophages from *Tlr2*$^{-/-}$, *Dect1*$^{-/-}$, and wild-type mice stimulated overnight with *C. albicans* of different morphological stages at ~10^6/ml.

(C) IL-1β responses of wild-type macrophages stimulated O/N with LPS at 500 ng/ml, β-glucan at 10 μg/ml, or zymosan at 1 μg/ml alone or primed for 6 hr and then removed and stimulated for an additional 8 hr by *C. albicans* at ~10^6/ml.

***p < 0.001; **p < 0.01; *p < 0.05. Error bars, ± SD.

supernatants by western blotting and found that only the cleaved IL-1β (i.e., the 17 kD mature form) could be detected in the supernatants (Figure 1C). Similar studies were performed with mouse bone marrow-derived macrophages (BMDM) (Figure 1D), which showed that *C. albicans*-induced IL-1β release was also strongest for the germ tube forms. Cleaved IL-1β could be detected in the supernatants by western blotting (Figure 1E).

The primary function of inflammasomes is to convert inactive pro-caspase-1 into the active, cleaved enzyme. Inflammasome complexes assemble upon activation by an appropriate stimulus, leading to the multimerization of the adaptor molecule ASC. Subsequently, pro-caspase-1 is recruited to ASC via the interactions of the CARD domains of ASC and caspase-1. The induced proximity of caspase-1 leads to autocleavage of caspase-1. The two resulting subunits, p10 and p20, assemble into the active caspase-1. The activation of caspase-1 was next examined by monitoring the cleavage status of caspase-1 in cell supernatants. To test whether *Candida* can directly activate the cleavage of caspase-1, we precipitated proteins from the supernatants of BMDM exposed to *C. albicans*. The caspase-1 p10 subunit could be detected in supernatants of *C. albicans* treated cells (Figure 1F). Together, these data indicate that *Candida* can activate caspase-1 to release bioactive IL-1β, suggesting that a caspase-1-activating inflammasome complex is being activated. The mechanism by which *C. albicans* triggers these events is unknown.

IL-1β Gene Transcription Is Dependent on TLR2 and Dectin-1

The CLR Dectin-1 is a receptor for fungal β-glucan and has been shown to cooperate with TLR2 for the transcriptional induction of inflammatory cytokine genes in response to *C. albicans* infection (Brown et al., 2002, 2003). First, we defined the role of TLR2 and Dectin-1 in the upregulation and activation of IL-1β in response to *C albicans*. Peritoneal macrophages from wild-type, TLR2, or Dectin-1-deficient mice were stimulated with *C. albicans*. As shown in Figure 2, *Candida* induced IL-1β mRNA upregulation (Figure 2A) and protein release into the supernatants (Figure 2B) from wild-type macrophages. These responses were completely abrogated in *Tlr2*$^{-/-}$ as well as *Dect1*$^{-/-}$ deficient macrophages, demonstrating the importance of these receptors in fungal-mediated IL-1β induction. Having established the role of *Candida* in the transcriptional induction of IL-1β, we next examined whether *Candida* could lead to the processing of pro-IL-1β followed by its release into supernatants. Macrophages were first exposed to the fungal cell wall PAMPs, β-glucan and zymosan, or to LPS and were then stimulated with *C. albicans*. Neither LPS, β-glucan, nor zymosan alone were sufficient to drive IL-1β release as shown in Figure 2C. In contrast, when macrophages were additionally treated with *C. albicans*, IL-1β release was detected. Importantly, IL-1β release was detected in cells exposed to *Candida* alone, and even higher levels of IL-1β were released in cells that had been exposed to β-glucan, zymosan, or LPS. These data indicate that ligands that trigger TLR2 and Dectin-1 signaling cannot by themselves elicit IL-1β processing and release. The ability of *Candida* to trigger these events in both unprimed (media-treated)

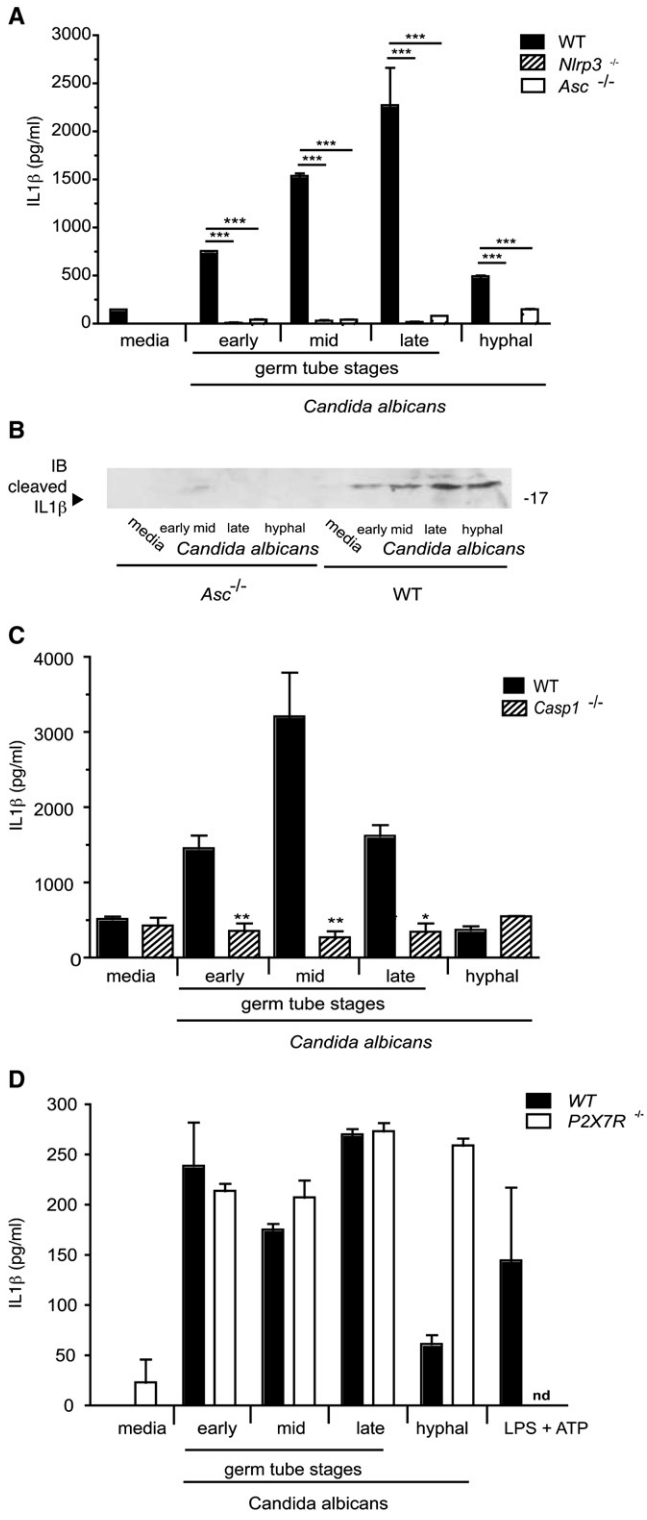

Figure 3. IL-1β Responses to *Candida albicans* Are Mediated by NLRP3, ASC, and Caspase-1

(A) IL-1β responses of macrophages from *Asc*$^{-/-}$, *Nlrp3*$^{-/-}$, and wild-type mice primed for 4 hr with 500 ng/ml LPS and then stimulated with *C. albicans* at ~10^6/ml.

(B) Western blot of supernatants from wild-type or *Asc*$^{-/-}$ macrophages probed with anti-IL-1β antibody.

(C) IL-1β responses of macrophages from *Casp1*$^{-/-}$ and wild-type mice stimulated overnight with *C. albicans* at ~10^6/ml.

(D) IL-1β responses of macrophages from *P2X7R*$^{-/-}$ and wild-type mice stimulated overnight with *C. albicans* at ~10^6/ml.

***p < 0.001; **p < 0.01; *p < 0.05. Error bars, ± SD.

and primed cells indicates that *Candida* can elicit IL-1β gene transcription (signal 1) and inflammasome activation (signal 2).

Candida albicans Triggers IL-1β Production via the NLRP3 Inflammasome

To define the molecular mechanism regulating caspase-1-mediated processing of IL-1β, we monitored inflammasome activation by *Candida* in LPS-primed macrophages. LPS-primed macrophages from wild-type mice or mice deficient in NLRP3 or ASC were stimulated with *C. albicans* and IL-1β release and cleavage examined by ELISA and western blotting, respectively. As shown in Figure 3A, IL-1β responses from macrophages deficient in NLRP3 and ASC were significantly reduced following treatment with all stages of *C. albicans*. LPS-primed macrophages exposed to ATP induced high levels of IL-1β responses in wild-type macrophages as well as in macrophages lacking TLR2 or Dectin-1, but not in macrophages deficient in ASC, NLRP3, or caspase-1 (Figures S2A and S2B), as expected. In contrast, induction of TNF-α and KC in response to *C. albicans* (which are dependent on TLR2/Dectin-1 signaling and not on the inflammasome) was comparable between wild-type and knockout cells (Figure S2C and data not shown). We also examined IL-1β cleavage in cellular supernatants by western blotting and found that the cleaved and active form of IL-1β could be detected in supernatants from wild-type, but not *Asc*$^{-/-}$ cells (Figure 3B). We also confirmed that *Candida*-induced IL-1β was dependent on caspase-1 (Figure 3C). To determine whether ATP was responsible for IL-1β production in response to *Candida*, macrophages from the ATP-gated ion channel receptor P2X7-deficient mice were stimulated with *C. albicans*. No difference in *Candida*-induced IL-1β responses between the P2X7R-deficient and wild-type cells were observed. As expected, a complete abrogation of LPS + ATP responses was seen (Figure 3D). Together, these data show that *C. albicans* is able to induce IL-1β gene transcription in a TLR2- and Dectin-1-dependent manner and that the processing of IL-1β is mediated by the NLRP3/ASC inflammasome.

IL-1β Controls Antifungal Immunity In Vivo

Having established the importance of TLR2, Dectin-1, and the NLRP3 inflammasome in controlling IL-1β production in macrophages, we assessed whether this pathway was important in vivo. We first examined whether *C. albicans* could induce IL-1β in vivo. To this effect, we established a murine model of mucosal infection to define the importance of IL-1β and IL-1 receptor signaling in vivo. To establish oral infection, we first treated mice with antibiotic containing drinking water to reduce the competing oral microflora. Next, a series of shallow scratches in the *stratum corneum* of the dorsal surface of the tongue were made in anesthetized mice using a sterile scalpel. Sterile PBS was applied for 3 hr to allow the disruptions to partially heal, and then concentrated *C. albicans* strain GDH2346 (NCYC 1467), originally obtained from a denture stomatitis patient at Glasgow Dental Hospital (McCourtie and Douglas, 1984), was

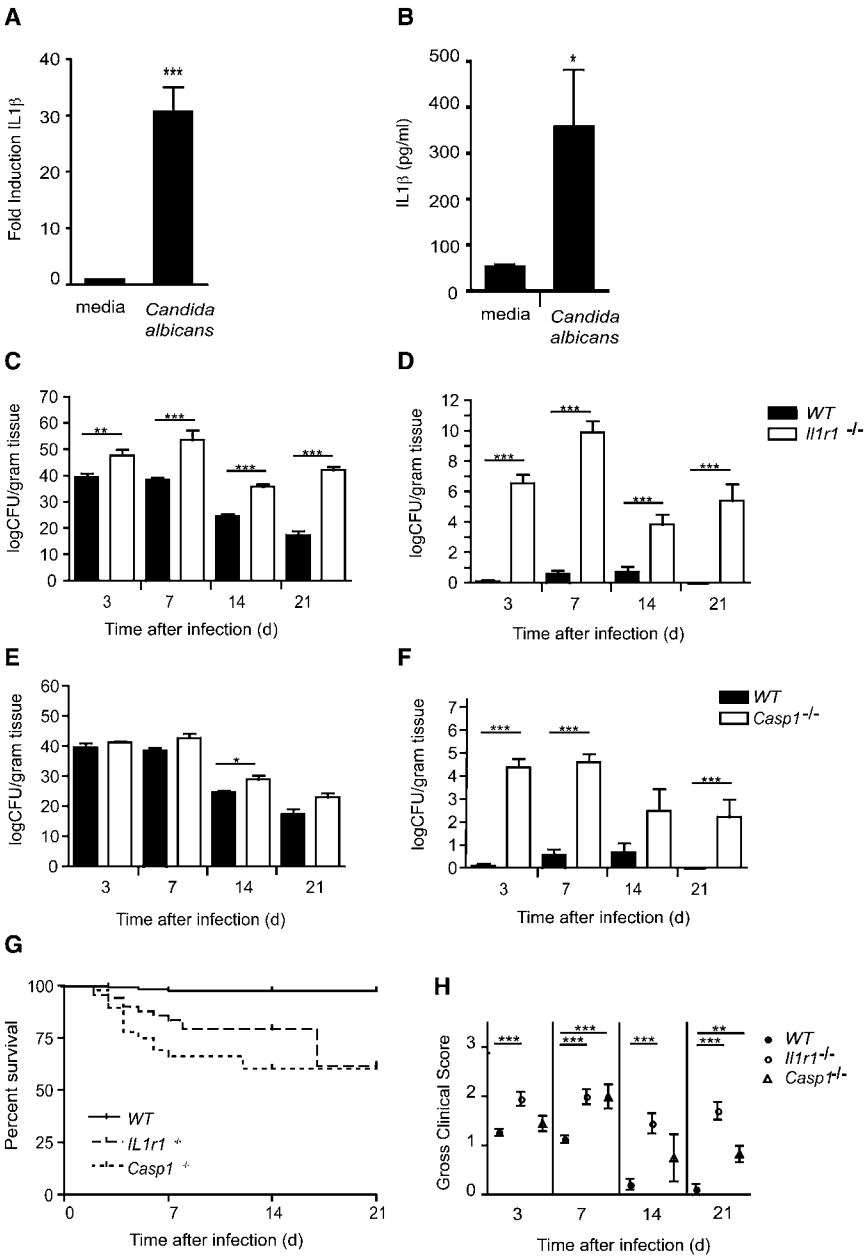

Figure 4. Protective Role of IL-1β in a Murine Model of Oral Infection with *Candida albicans*

(A) Fold induction of IL-1β mRNA measured by quantitative real-time PCR by oral buccal epithelium after infection with *C. albicans*.

(B) IL-1β (pg/ml) protein production in homogenized whole tongues of mice after infection with *C. albicans*.

(C and D) Quantitative fungal burden of (C) tongues and (D) kidneys of *Il1r1*$^{-/-}$ and wild-type (WT) mice after oral infection with *C. albicans*.

(E and F) Fungal burden of (E) tongues and (F) kidneys of *Casp1*$^{-/-}$ and WT mice after oral infection with *C. albicans*.

(G) Kaplan-Meier survival plots of WT, *Il1r1*$^{-/-}$, and *Casp1*$^{-/-}$ mice after infection ($p < 0.0001$).

(H) Mean clinical severity score of *Il1r1*$^{-/-}$, *Casp1*$^{-/-}$, and wild-type after 3, 7, 14, or 21 days of infection.

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Error bars, ± SD.

applied to the oral cavity and allowed to remain in a saturated cotton ball for 4 to 6 hr with the animals under sedation, allowing for consistent and sustained colonization of the oral mucosa (Figure S3). There was no difference seen in the severity of infection, as determined by clinical scores, between mice infected with and without disruption of the *stratum corneum*, but we found that the additional disruption step led to more consistent infections with little variation in fungal colonization of the oral cavity between individual mice. Although colonization of the tongue was slightly lower in undisrupted mice as measured by fungal culture, there was no difference in colonization of the gut (esophagus, stomach, duodenum, jejunum, and ileum) or kidneys (a marker for systemic dissemination), as shown in Figure S5. Wild-type mice were infected and the epithelium harvested from the buccal surfaces for mRNA isolation and protein extraction. As shown in Figure 4, both IL-1β mRNA (Figure 4A) and protein (Figure 4B) levels were strongly induced in vivo in oral mucosa by *C. albicans* infection.

To define the importance of IL-1β in mucosal fungal infection, wild-type, or IL-1 receptor-deficient mice (*Il1r1*$^{-/-}$) were infected orally with *C. albicans*. After 3, 7, 14, and 21 days of infection, mice were euthanized and clinically scored. Tongues and organs were removed aseptically and quantitative fungal burdens of tongues and kidneys determined. As shown in Figure 4C, fungal colonization of the oral cavity was significantly higher in the *Il1r*$^{-/-}$ mice at all time points. Neither wild-type nor *Il1r*$^{-/-}$ mice cleared the oral infection during the study period. In contrast to wild-type mice, the *Il1r*$^{-/-}$ mice showed dramatically higher levels of systemic dissemination, as measured by quantitative fungal burdens in the kidneys, at all time points measured (Figure 4D). Survival was also impaired in the *Il1r*$^{-/-}$ mice with cumulative 63.7% survival after 21 days compared to 97.0% in the wild-type mice (Figure 4G). Clinical severity, as assessed by gross scores at the experimental endpoint (Figure S3), was also significantly higher in *Il1r*$^{-/-}$ mice (Figure 4H).

We also investigated the role of caspase-1 by infecting *Casp1*$^{-/-}$ or wild-type mice with *C. albicans* as above. As shown in Figure 4E, colonization of the oral cavity was similar between the *Casp1*$^{-/-}$ and wild-type mice at all time points but slightly higher at 14 days. Both strains failed to clear the oral infection during the study period. In contrast to oral colonization, the *Casp1*$^{-/-}$ mice showed greatly enhanced systemic dissemination, as measured by quantitative fungal burdens in the kidneys, with significance at 3 and

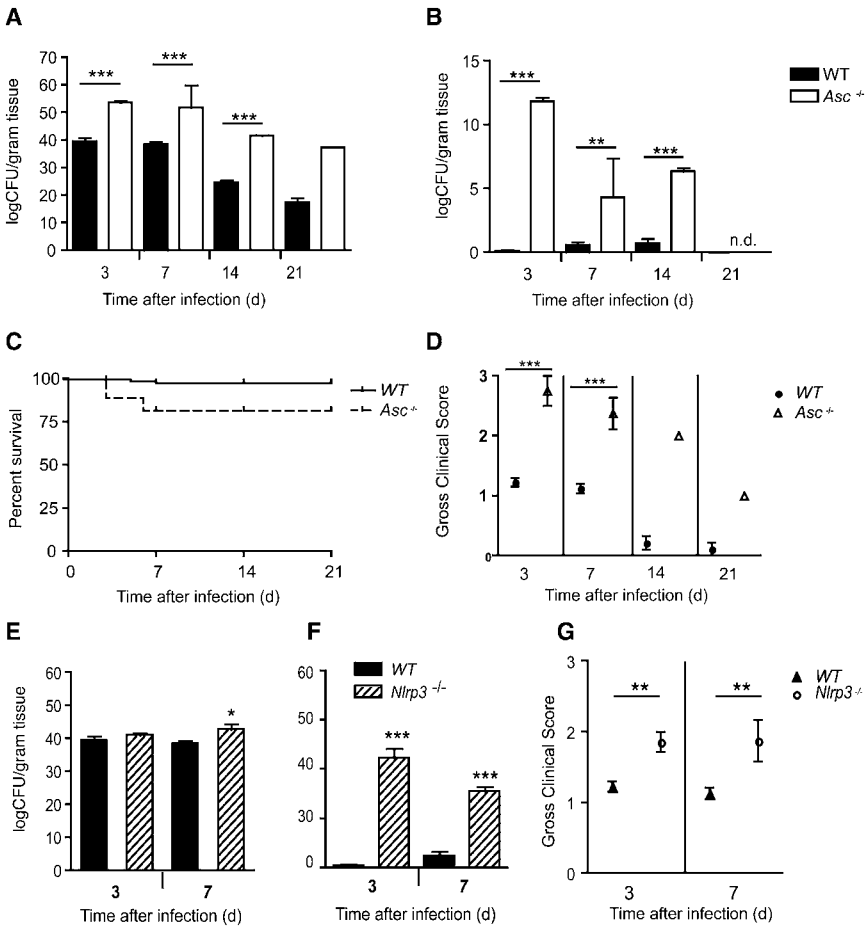

Figure 5. NLRP3 Inflammasome Plays a Critical Role in Host Defense to Mucosal Infection with *Candida albicans*

(A and B) Quantitative fungal burden of (A) tongues and (B) kidneys of *Asc*$^{-/-}$ and WT mice after oral infection with *C. albicans*.

(C) Kaplan-Meier survival plots of WT and *Asc*$^{-/-}$ mice after infection ($p = 0.0002$).

(D) Mean clinical severity score of *Asc*$^{-/-}$ and WT after 3, 7, 14, or 21 days of infection.

(E and F) Quantitative fungal burden of (E) tongues and (F) kidneys of *Nlrp3*$^{-/-}$ and WT mice after oral infection with *C. albicans*.

(G) Mean clinical severity score of *Nlrp3*$^{-/-}$ and WT after 3 and 7 days of infection.

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Error bars, ± SD.

7 days and a trend toward significance at 14 and 21 days (Figure 4F). Survival was also impaired in the *Casp1*$^{-/-}$ mice, with cumulative 60.3% survival after 21 days compared to 97.0% in the wild-type mice (Figure 4G). Clinical severity, as assessed by gross scores at the experimental endpoint, was also significantly higher in *Casp1*$^{-/-}$ mice compared to wild-type mice at all time points (Figure 4H).

The NLRP3 Inflammasome Controls Antifungal Immunity In Vivo

Because ASC and NLRP3 control IL-1β production in response to *C. albicans* in macrophages, we next tested the importance of the NLRP3 inflammasome complex in antifungal defense in vivo. As shown in Figure 5A, mice deficient in ASC had higher oral colonization after infection with *C. albicans*, as measured by quantitative fungal burden of homogenized tongues. This correlated with higher gross clinical scores in the *Asc*$^{-/-}$ mice compared to wild-type (Figure 5D). Also, functional ASC is required for resistance to dissemination of infection, as shown in Figure 5B, with significantly higher fungal burdens in the kidneys compared with wild-type mice. Survival was also significantly affected in the ASC-deficient mice compared to wild-type (Figure 5C). NLRP3-deficient mice infected with *C. albicans* showed similar oral colonization as compared to wild-type mice but showed enhanced dissemination with significantly higher fungal burdens in the kidneys at 3 and 7 days of infection. Clinical severity of infection was also worse in the NLRP3-deficient mice (Figure 5G). We hypothesize that systemic dissemination of *C. albicans* occurs via translocation or invasion of the fungus through the mucosa of the small intestine, which has been demonstrated in some models (Cole et al., 1988). To assess the impact of the NLRP3 inflammasome on intestinal colonization, organs were harvested and quantitatively cultured for fungal colonization. Both *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice demonstrated higher fungal burdens of the stomach, duodenum, jejunum, and ileum at 7 days of infection compared to wild-type mice, which appear to partially clear infection of the small bowel (Figure S4). Together, using a model of mucosal candidiasis, we show that activation of the NLRP3 inflammasome by *C. albicans* affects colonization of the gut, dissemination of infection, and survival in OPC.

TLR2 and Dectin-1 Control Antifungal Immunity In Vivo

Because TLR2 and Dectin-1 regulated IL-1β production in response to *C. albicans*, we also determined the role of TLR2 and Dectin-1 in the murine model. As shown in Figure 6A, oral colonization was similar between wild-type and Dectin-1-deficient mice, despite higher clinical scores (Figure 6D). However, dissemination of infection occurred in the *Dect1*$^{-/-}$ mice (Figure 6B), as did significantly reduced survival (Figure 6C). In TLR2-deficient mice, both enhanced oral colonization and dissemination of infection were observed (Figures 6E and 6F). TLR2-deficient mice also had significantly less survival and higher clinical scores than wild-type mice. Therefore, both TLR2 and Dectin-1 play an important role in host defense to mucosal infection with *C. albicans*.

TLR2, Dectin-1, and the NLRP3 Inflammasome Control IL-1β Production In Vivo

Finally, circulating levels of IL-1β in serum from mice infected orally with *C. albicans* were quantified in all of these strains. Serum IL-1β responses to *C. albicans* infection were partially reduced in *Tlr2*$^{-/-}$ mice and completely abolished in *Dect1*$^{-/-}$ or double *Dect1*/*Tlr2*$^{-/-}$ mice, indicating a cooperative role for

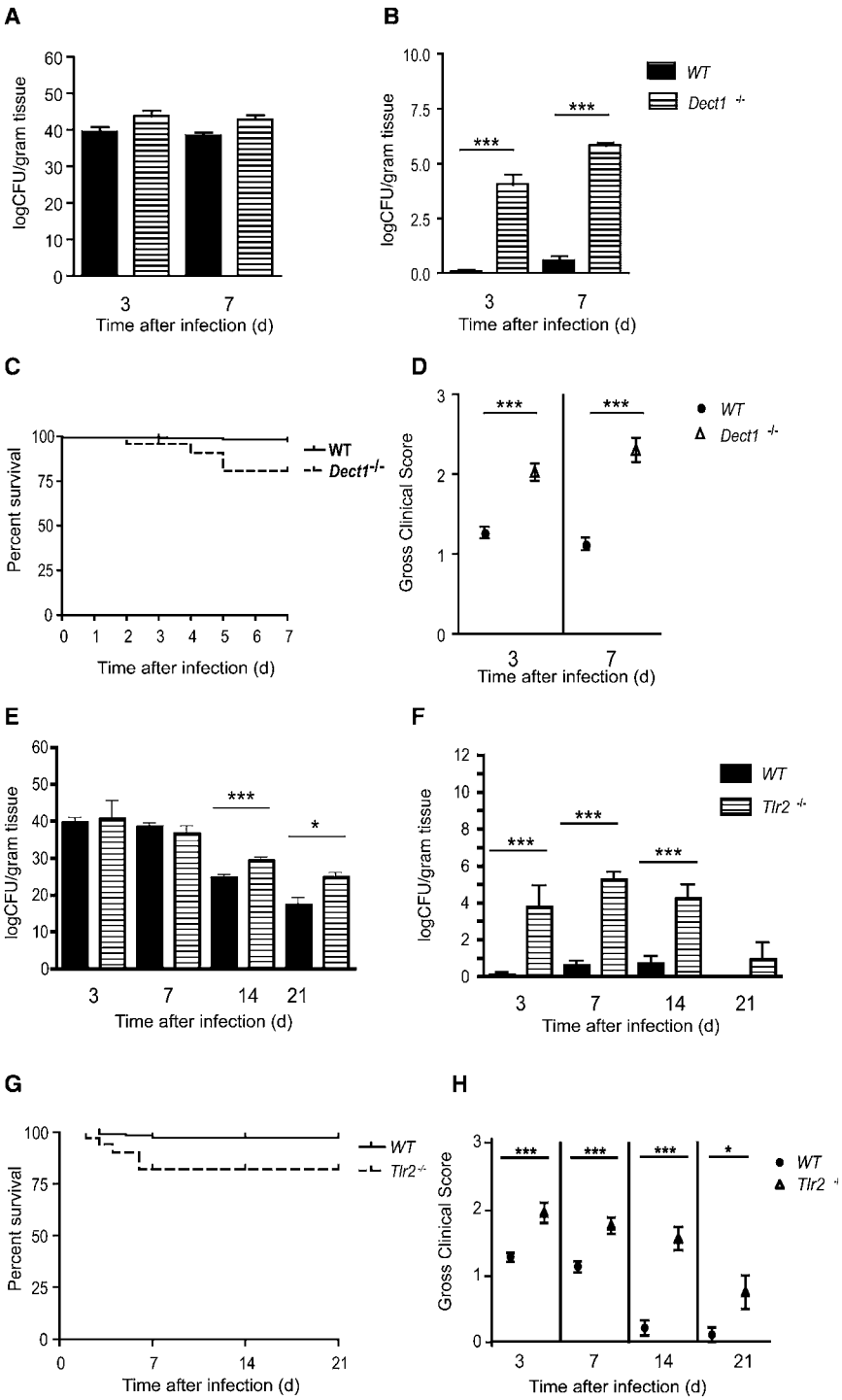

Figure 6. Innate PRRs Enhance Survival after Oral Infection with *Candida albicans*

(A and B) Quantitative fungal burden of (A) tongues and (B) kidneys of *Dect1*$^{-/-}$ and wild-type (WT) mice after oral infection with *C. albicans*.

(C) Kaplan-Meier survival plots of WT and *Dect1*$^{-/-}$ mice after infection ($p = 0.0009$).

(D) Mean clinical severity score of *Dect1*$^{-/-}$ and WT after 3 or 7 days of infection.

(E and F) Quantitative fungal burden of (E) tongues and (F) kidneys of *Tlr2*$^{-/-}$ and wild-type (WT) mice after oral infection with *C. albicans*.

(G) Kaplan-Meier survival plots of WT and *Tlr2*$^{-/-}$ mice after infection ($p = 0.0002$).

(H) Mean clinical severity score of *Tlr2*$^{-/-}$ and WT after 3, 7, 14, or 21 days of infection.

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Error bars, ± SD.

these receptors in regulating IL-1β production in vivo (Figures 7A and 7C). IL-1β production was undetectable in serum from *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice (Figure 7B).

DISCUSSION

Oral epithelial cells respond to fungal pathogens with the release of proinflammatory cytokines, including IL-1α/β, IL-8, TNF-α, and antimicrobial peptides such as β-defensins (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou et al., 2004; Lu et al., 2006; Mostefaoui et al., 2004). Although encoded by different genes, IL-1α and IL-1β share highly homologous structures and activate through the common receptor IL-1 receptor 1 (IL-1R1), which functions as a heterodimer with the IL-1 receptor accessory protein (IL-1RAcP) and signals via the TIR domain-containing adaptor molecule MyD88 (Dinarello, 1998). Both IL-1α and IL-1β, as well as IL-18, have been suggested to play an important role in host defense against *C. albicans* infections (Bellocchio et al., 2004; Mencacci et al., 2000; Stuyt et al., 2002, 2004; Vonk et al., 2006), although none have direct antifungal activity. IL-18, which is also processed by NLRs, has been shown to be upregulated in a caspase-1-dependent manner in response to *Candida* as well as mediating the development of protective Th1 immunity (Mencacci et al., 2000). It is likely that the NLRP3 inflammasome controls the processing of both IL-1β and IL-18 in response to fungal pathogens, as has been seen in the case of other activators of this inflammasome (Akita et al., 1997; Fantuzzi and Dinarello, 1999; Ghayur et al., 1997; Gu et al., 1997; Kuida et al., 1995).

Our studies reveal that *C. albicans* triggers pathways leading to the production of IL-1β. Further, we show that it acts both (1) as an inducer of pro-IL-1β (signal 1), as determined by measuring IL-1β mRNA levels (Figure 2) and secretion of IL-1β in naive cells, a response that was dependent on both TLR2 and Dectin-1 and (2) as an activator of the NLRP3 inflammasome to process pro-IL-1β (signal 2). The ability of fixed preparations to elicit this response suggests that fungal invasion and/or

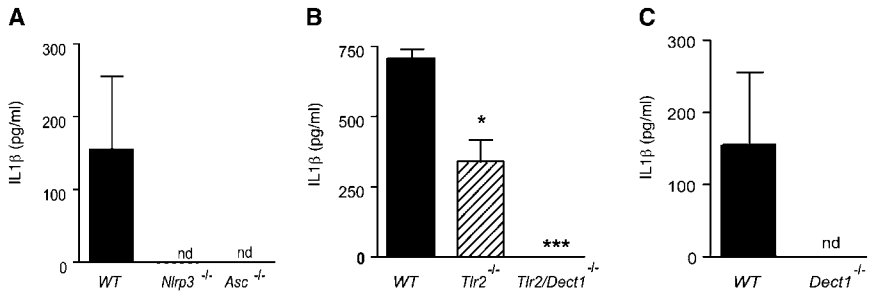

Figure 7. Serum IL-1β Responses to In Vivo Infection with *C. albicans* Are Dependent on TLR2, Dectin-1, and the NLRP3 Inflammasome

(A) IL-1β responses in serum collected from wild-type, *Nlrp3*$^{-/-}$, or Asc$^{-/-}$ mice orally infected with *C. albicans*.

(B) IL-1β responses in serum collected from orally infected wild-type, *Tlr2*$^{-/-}$, or double *Tlr2/Dect1*$^{-/-}$ mice.

(C) IL-1β responses in serum collected from orally infected wild-type or *Dect1*$^{-/-}$ mice.

***p < 0.001; *p < 0.05. Error bars, ± SD.

metabolic activity are not required. We utilized killed preparations for our in vitro studies in an effort to avoid IL-1β release due to *Candida*-induced cell toxicity and death (which we did not observe in our in vitro studies). As shown previously, heat inactivation of *Candida* can unmask β-glucan structures that bind Dectin-1 (Gantner et al., 2005). To avoid this, we used formalin fixation to kill the organism without causing artificial exposure of β-glucans.

Our data show that both early germ tube and fully hyphal forms of *Candida* are capable of inducing IL-1β release in human PBMC and THP-1 cells. Release of cleaved functional IL-1β is confirmed by immunoblot analysis for the 17 kDa active cytokine. Moreover, release of IL-1β, cleavage of IL-1β, and cleavage of caspase-1 were also observed in both peritoneal and bone marrow-derived macrophages from mice. The differential ability of the different forms of *Candida* to trigger signaling is consistent with published data on differential immune activation by different morphological stages of fungus (Bellocchio et al., 2004; Gantner et al., 2005). It is unclear why the studies recently published by another group did not reveal a role for the inflammasome in the production of IL-1β; however, there are substantial methodological differences between the two studies (van de Veerdonk et al., 2009). We show a profound suppression of IL-1β production in macrophages from NLRP3-deficient, ASC-deficient, and caspase-1-deficient mice. Most importantly, our studies using a mucosal model of *Candida* challenge show a significant phenotype in the inflammasome-deficient mice, providing in vivo support to our in vitro findings. Altogether, these data implicate that the inflammasome is activated and that NLRP3, ASC, and caspase-1 mediate *Candida*-induced IL-1β responses and antifungal defense both in vitro and in vivo. A recent publication using an intravenous challenge model and in vitro studies also supports a role for NLRP3 in IL-1β responses to *Candida* and is consistent with our findings (Gross et al., 2009).

Both TLR2 and dectin-1 are known to play an important role in fungal recognition and the induction of inflammatory responses. Dectin-1, which recognizes β-glucan in the fungal cell wall, has been shown to mediate phagocytosis of fungal pathogens in a partially Syk-dependent manner, depending on the cell type studied (Herre et al., 2004; Kerrigan and Brown, 2009; Rogers et al., 2005). Although TLR2 recognizes fungal PAMPs and mediates inflammatory responses, it is not required for phagocytosis; macrophages lacking TLR2, TLR4, or MyD88 internalize yeast particles normally (Gantner et al., 2003). We show that the fungal PAMPs β-glucan and zymosan, which bind Dectin-1 and TLR2, respectively, can prime cells for subsequent IL-1β release by *C. albicans* but by themselves are unable to elicit this response. *C. albicans* can therefore elicit pro-IL-1β induction and cleavage. This is achieved by its ability to trigger TLR2 and dectin-1 and activate the NLRP3 inflammasome to cleave pro-IL-1β into its biologically active form. IL-1β was induced by inactive forms of the fungi, indicating again that neither metabolic activity nor active invasion of cells was required. Early germ tube or hyphal forms were generally more stimulatory than the fully hyphal form in vitro. Therefore, it is likely that there is differential activation by *C. albicans* in the context of biofilms (i.e., fully hyphal forms) that colonize the outer layers of epithelium, as compared to yeast and early germ tube morphological forms more often associated with extravasating or early disseminated infection (Saville et al., 2003, 2008).

We developed a murine model of OPC in which immunosuppression is not required and animals develop the characteristic white patches and epithelial ulceration seen in humans with oral thrush. Our goal was to establish a model of sustained mucosal colonization that would closely mimic human oropharyngeal candidiasis. Histologically, the infections that we achieve are similar to human disease (Figure S3). Our model is also unique, as we observe low-level, chronic dissemination from a mucosal source in susceptible strains, which has not previously been modeled.

Using this model, we have defined an essential role for IL-1β in host defense to *Candida* infection. Increased fungal burdens were seen in the tongues of IL-1 receptor-deficient mice. Moreover, dissemination or invasion of infection from the oral source was also dependent on IL-1β, with higher kidney fungal burdens (a marker for dissemination) and decreased survival in *Il1r*$^{-/-}$ mice. *Candida* can also be cultured from multiple organs in susceptible strains, including the brain, lung, liver, bowel, stomach, and spleen (data not shown). Using this physiologically relevant model of sustained oral infection, we also reveal the importance of TLR2 and Dectin-1, as well as the NLRP3 inflammasome (caspase-1, ASC, and NLRP3), in controlling IL-1β production, which determines resistance against dissemination and promotes survival of the infected host.

Key questions arising from our studies are how NLRP3 senses *C. albicans* and what component of the pathogen triggers the NLRP3 inflammasome. Numerous chemically and structurally diverse stimuli are now known to activate the NLRP3 inflammasome. These include pathogenic bacteria and viruses, as well as a broad range of microbial derivatives, bacterial pore forming toxins, small molecule immune activators, extracellular ATP, amyloid-β, and various crystals (e.g., silica and asbestos) (Halle

et al., 2008; Hornung et al., 2008; Kanneganti et al., 2006a, 2006b; Martinon et al., 2004, 2006). It remains unclear, however, how a single signaling molecule NLRP3 can detect such a broad range of stimuli. Because there is no evidence that all of these ligands bind directly to NLRP3, it has been suggested that activation is indirect. Our studies with P2X7R-deficient macrophages indicate that ATP release from *Candida*-infected cells is not responsible for triggering NLRP3 activation. The generation of reactive oxygen species and cathepsin B release from damaged lysosomal compartments are additional proposed mechanisms for activation of the NLRP3 inflammasome. The contribution of cathepsin B has been found to be specific to NLRP3 activation by particulates such as silica and amyloid-β. Phagocytosis and lysosomal acidification are believed to be important for NLRP3 activation by these agents. Additional work is needed to elucidate the role of phagocytosis, lysosomal disintegration, and ROS generation in activation of the NLRP3 inflammasome by *C. albicans*.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6 and *Il1r*$^{-/-}$ mice (ages 8–12 weeks) were purchased from Jackson Laboratories. *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice were generated by Millenium Pharmaceuticals. *Casp1*$^{-/-}$ mice were generated by R. Flavell (Yale University). *P2X7R*$^{-/-}$ mice (C57BL/6 background) were originally provided by Pfizer Global Research and Development, Pfizer Inc. (Solle et al., 2001) and then backcrossed into a pure C57BL/6 background for > 12 generations from a *P2X7R*$^{-/-}$ mouse strain. Animals were housed in filter-covered microisolator cages in ventilated racks. All animal studies have been approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and University of Massachusetts.

Reagents

Cells were cultured in RPMI 1640 media (HyClone) containing 10% non-HI FCS (Atlanta Biologicals). β-glucan (isolated from *Saccharomyces cerevisiae*) and Z-YVAD-fmk were purchased from CalBiochem. Zymosan was obtained from Sigma. Macrophages were lysed using Radio-Immunoprecipitation Assay Buffer (Pierce) containing Halt Protease Inhibitor Cocktail (Thermo Scientific). Western blots were probed using monoclonal 3ZD anti-IL-1β antibody (National Cancer Institute Biological Resources Branch) and polyclonal anti-capase-1 p10 antibody (Santa Cruz Biotechnology, Inc).

Fungal Preparations

Candida albicans strain GDH2346, clinical strain originally isolated from a denture stomatitis patient (McCourtie and Douglas, 1984), was utilized for all in vivo and in vitro studies. Stocks were maintained on Sabouraud Dextrose (SD) agar at 4°C. Prior to challenge in the animal model, a suspension was made by transferring the fungus to SD broth (Difco) and incubating at 37°C with shaking for a period of 16–24 hr. To obtain hyphal and intermediate germ tube forms, we grew the *Candida* in enriched media consisting of RPMI + 10% fetal bovine serum (FBS) for 2, 4, or 6 hr (early, mid, or late germ tube formations) or for 24 hr for fully hyphal forms (Figure S6). For formaldehyde fixation, the preparations were washed in sterile PBS, fixed in 1% formaldehyde solution for 10 min, and then washed three times in sterile PBS. After resuspension in sterile PBS, the preps were stored at −80°C until use.

Cell Isolation and Culture

Bone marrow-derived macrophages were generated as described (Severa et al., 2006). Human PBMCs were isolated from whole blood of healthy volunteers by density gradient centrifugation. Lysis of red blood cells was performed using red blood cell lysis buffer (Sigma). Experiments in PBMCs and macrophages were carried out at a cell density of 2 × 10^6 cells/ml. All primary cells and cell lines except THP-1 cells were cultured in DMEM supplemented with L-glutamine, ciprofloxacin (Cellgro), and 10% fetal calf serum (Hyclone). THP-1 cells were cultured in RPMI supplemented with 10% fetal calf serum (Hyclone), L-glutamine, sodium pyruvate (Cellgro), and ciprofloxacin. At 1 day prior to stimulation, THP-1 cells were differentiated using 0.5 μM PMA for 3 hr, washed three times, and plated for stimulation. The caspase-1 inhibitor z-YVAD (10 mM) was added for 1 hr prior to stimulation. Thioglycollate-elicited peritoneal macrophages were isolated by adherence and stimulated at a density of 2 × 10^6 cells per well in a 6-well plate in RPMI 1640 (HyClone) with 10% FCS (Atlanta Biologicals). Cells were primed with 200–500 ng/ml low-protein LPS for 3–4 hr, followed by fixed *Candida* at ~10^6/ml for 8 hr. Alternatively, naive cells were stimulated overnight with fixed *Candida* preparations. Supernatants were harvested and assayed for cytokine release. Cells were lysed in RIPA buffer and run on SDS-PAGE gel and transferred to nitrocellulose, followed by probing for IL-1β and caspase-1. All experiments that were performed for western blot analysis were carried out in serum-free DMEM medium. ATP stimulations were carried out at 5 mM 1 hr prior to harvesting supernatants.

Murine Model of OPC

Five days prior to challenge with *C. albicans*, the mice were placed on tetracycline (Fisher Scientific) containing drinking water (2.5 g/l). The mice were anesthetized i.p. with a cocktail of Ketamine (Vedco) and Acepromazine (Boehringer Ingelheim), and the dorsal surface of the tongue was lightly scratched (limited to the superficial stratum corneum of the epithelial layer) using a sterile #10 blade scalpel to roughen the surface. A sterile, uniform-sized cotton packing was placed in the oral cavity and saturated with PBS to keep the mouth moist. After 3 hr, the cotton was replaced and saturated with 100 μl of 5.0 × 10^7/ml CFU suspension of *Candida albicans* yeast in PBS and left in place for ~4–6 hr while the animals remain sedated. After a 3–28 day infection period, the mice were given a clinical score to assess the degree of oral infection (Figure S3); the tongues and other target organs were collected aseptically after euthanasia, weighed, and homogenized in sterile-normal saline using a TissueLyser bead-beater homogenizer (Retsch) in individual sterile nuclease-free tubes. Serial dilutions of the homogenate (10-fold) were made in sterile PBS and plated in triplicate onto SD agar, sealed and incubated for 48 hr at 37°C, and fungal colonies manually counted. Fungal burdens of the tissues are shown as log CFU per gram of tissue (absolute fungal data is included in Table S1). To assess inflammatory responses in the oral mucosa, buccal epithelium was removed at necropsy by blunt dissection and placed in RNAlater (QIAGEN) or PBS, homogenized, and frozen at -80°C for mRNA isolation or protein assay, respectively. For serum collection, whole blood was obtained from retro-orbital sinus into EDTA precoated tubes and was centrifuged, and serum was removed and frozen.

Quantitative Real-Time PCR and Enzyme-Linked Immunosorbent Assay

Quantitative real-time PCR was done as described (see Supplemental Experimental Procedures for additional details). Cytokines were measured in culture supernatants collected after 24 hr of stimulation by ELISA (R&D).

Western Blot Analysis

Cell culture supernatants were precipitated by adding an equal volume of methanol and 0.25 volumes of chloroform, mixed and centrifuged at 20,000 × g for 10 min. The upper phase was discarded, and 500 μl of methanol was added to the interphase. This mixture was centrifuged at 20,000 × g for 10 min and the protein pellet dried at 55°C, resuspended in Laemmli buffer, and boiled at 99°C for 5 min. Samples were separated by SDS-PAGE (15%) and transferred onto nitrocellulose membranes. As indicated, blots were incubated with rabbit polyclonal antibody to anti-murine caspase-1 p10 (sc-514, Santa Cruz Biotechnology), rabbit polyclonal anti-human caspase-1 p10 (sc-515, Santa Cruz Biotechnology), rabbit polyclonal anti-human cleaved IL-1β (Asp116) (Cell Signaling), or rabbit polyclonal anti-murine cathepsin B (R&D Systems).

Statistical Analysis

Data were analyzed using commercial software (GraphPad Software), and Student's two-sample independent t test was used for statistical analysis. Comparison of two survival curves was done using the Logrank test. P values are presented where statistical significance was found (significance set at $p < 0.05$).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00143-7.

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